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Glucosamine promotes chondrogenic phenotype in both chondrocytes and mesenchymal stem cells and inhibits MMP-13 expression and matrix degradation

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Summary

Objectives: Glucosamine (GlcN), a natural amino monosaccharide, is a constituent of glycosaminoglycans (GAGs) found in hyaline cartilage. GlcN salts constitute a new class of nutraceutical components with putative chondroprotective activity, which may target chondrocytes as well as chondroprogenitors cells, such as mesenchymal stem cells (MSCs), during cartilage turnover and repair. In the present study, we examined the effects of GlcN on chondrogenesis of human MSCs (hMSCs) and the phenotype of normal and osteoarthritic human articular chondrocytes, using an *in vitro* pellet culture model maintained in a defined medium.

Methods: hMSCs and normal and osteoarthritic human chondrocytes grown as pellet cultures, stimulated or not with interleukin-1β (IL-1β), were treated with varying doses of GlcN. Expression of cartilage matrix genes and cartilage degrading enzymes was determined by semiquantitative and quantitative real-time reverse transcription polymerase chain reaction (RT-PCR), and by histological staining of cartilage markers, as well as sulfated GAG (sGAG) analysis and Western blotting.

Results: Chondrocytes grown in the presence of serum for 11 days showed decreased expression of the cartilage matrix genes, collagen type II (collagen II) and aggrecan, as early as day 3, which was reversed with GlcN treatment by day 11. Both hMSCs and chondrocytes grown as pellet cultures in defined medium and treated with 100 μM GlcN exhibited enhanced expression of collagen II and aggrecan as well as increased content of sGAG, when compared to control untreated pellets. However, high doses of GlcN (10–20 mM) were inhibitory. GlcN treatment partially blocked IL-1β mediated downregulation of collagen II and aggrecan expression and inhibited expression of the matrix degrading enzyme, matrix metalloproteinase 13 (MMP-13), in both chondrocytes and hMSCs undergoing chondrogenesis.

Conclusions: These observations suggest that GlcN treatment enhances hMSC chondrogenesis and maintains cartilage matrix gene expression in chondrocytes, which may account for some of the reported chondroprotective properties of GlcN on cartilage.

Key words: Glucosamine, Osteoarthritis, Mesenchymal stem cells, Chondrocytes, Matrix metalloproteinase 13, Cartilage.

Introduction

Articular cartilage, located at the end of long bones, consists of chondrocytes embedded in a hyaline cartilaginous extracellular matrix (ECM). The major components of the articular cartilage ECM are collagen type II (collagen II) and proteoglycans, and the organization and the homeostasis of the tissue is maintained via a balance of anabolic and catabolic functions. Osteoarthritis (OA) is a degenerative joint disease of the articular cartilage associated with multiple factors, genetic, metabolic, biochemical and biomechanical, that act to disturb the equilibrium of anabolic and catabolic events, resulting in the depletion of collagens and proteoglycans due in part to accelerated turnover and inadequate repair[1,2]. In addition, local inflammatory activity is a well-known component of OA[3]. Articular cartilage is an avascular tissue that interacts with circulating factors through the surrounding synovium and subchondral bone. Interleukin-1β (IL-1β), a pro-inflammatory cytokine released by synovial fibroblasts, is well known to increase locally during the osteoarthritic process[4]. IL-1β has been shown to directly depress chondrocyte synthesis of aggrecan and collagen[5–7]. In addition, IL-1β activates a large cascade of mediators, such as matrix metalloproteinases (MMPs), that play an active role in ECM degradation, leading to cartilage damage[8].

Current pharmacological treatments of arthritis are limited to analgesics such as oral nonsteroidal anti-inflammatory drugs (e.g., ibuprofen), injectable intra-articular corticosteroids (betametazone) and hyaluronic acid, which control disease symptoms and provide pain relief but do not affect the progression of the disease. Glucosamine (GlcN) is an amino monosaccharide found naturally in connective tissues and is a chemical constituent of glycosaminoglycans (GAGs), building blocks of the large aggregating proteoglycans of the cartilage ECM[9,10]. GlcN has been touted for more than two decades as an effective nutraceutical for the purpose of chondroprotection. Several clinical trials that explored the potential of GlcN and another amino

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monosaccharide, chondroitin sulfate, in the treatment of OA have been recently reviewed, and some efficacy of GlcN in preventing joint space loss and relieving pain is suggested[11–14]. In human patients, administration of 1500 mg GlcN/day for 3 years prevented joint space loss and relieved symptoms when compared to placebo, suggesting that GlcN possesses some anti-inflammatory activity that can alter disease progression[15]. More recently, a GlcN/chondroitin arthritis intervention trial (GAIT) at 16 sites across the United States showed that participants with moderate to severe pain experienced significant pain relief with the combined supplements[16]. *In vitro*, GlcN treatment of human OA chondrocytes pretreated with IL-1β resulted in reduced activation of cartilage catabolic mediators, such as synthesis of nitric oxide (NO) and prostaglandin E_2, and enhanced aggrecan protein levels[17–19], suggesting that GlcN may act by controlling inflammatory mediators and stimulating proteoglycan synthesis in response to cartilage damage.

Articular cartilage is known for its poor capacity for self-repair, likely associated with the absence of blood supply, low mobility and a limited number of progenitor cells. Adult progenitor cells, also known as mesenchymal stem cells (MSCs), have been isolated from multiple human adult tissues including bone marrow, bone, muscle, tendon and adipose tissues. These cells have the capacity to self-renew for a limited number of population doublings and to exhibit the potential for multilineage differentiation, including chondrogenesis. Interestingly, culture expanded human OA chondrocytes grown as high-density pellet cultures in defined medium containing ascorbate and treated with dexamethasone (DEX) and transforming growth factor-β (TGF-β), formed cartilage with no signs of bone[20]. These findings suggest that diseased chondrocytes are able to revert to their normal differentiated phenotype. In addition, MSCs were recently identified in human cartilage and their frequency was increased in OA cartilage[21]. Taken together, these observations suggest that the activity of these cells is important in the progression of OA and other degenerative joint diseases. We postulate that GlcN may act by altering both chondrocyte and adult mesenchymal progenitor cell function during cartilage turnover and repair.

To test this hypothesis, we have examined the effects of GlcN on human OA articular chondrocytes, activated with IL-1β. In addition, we have investigated the effect of GlcN on the differentiation characteristics of multipotential, human MSCs (hMSCs). These cells can differentiate into chondrogenic phenotype, when cultured as high-density cell pellets in defined medium supplemented with DEX and TGF-β. We report here that GlcN treatment enhanced chondrogenic phenotype of hMSCs and the chondrogenic phenotype of OA chondrocytes, as shown by upregulation of the expression of cartilage matrix components, collagen II and aggrecan, and increased the levels of sulfated GAG (sGAG). GlcN treatment also blocked IL-1β-induced MMP-13 expression in both normal and OA chondrocytes, and enhanced aggrecan levels in OA chondrocytes following IL-1β challenge. These results strongly suggest that GlcN enhances chondrogenesis of hMSCs and maintains the chondrogenic phenotype of normal and OA chondrocytes.

Materials and methods

MATERIALS

DEX and GlcN hydrochloride (GlcN-HCl) were purchased from Sigma (Sigma, St. Louis, MO) and were dissolved in water as 1 mM and 100 mM stock solutions, respectively. Recombinant human TGF-β3 was from R&D systems (Minneapolis, MN) and dissolved in 4 mM HCl containing 0.1% bovine serum albumin (BSA) as a 2 μg/ml stock solution. IL-1β (R&D systems, Minneapolis, MN) was dissolved as a 5 μg/ml stock solution in phosphate buffer saline (PBS) containing 0.1% BSA.

INDUCTION OF CHONDROGENESIS IN hMSCs

A multipotential hMSC cell line previously established in our laboratory was used in this study[22–24]. The cell line was derived from human adult trabecular bone and stably transduced with human papilloma virus (HPV) oncoproteins E6/E7. hMSCs were maintained as monolayer cultures in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (Biowhittaker, Walkersville, MD), containing L-glutamine, and supplemented with 10% of specific lots of fetal bovine serum (FBS) (Atlanta Biological, Atlanta, GA) and antibiotic–antimycotic solutions (10,000 U Penicillin, 10,000 μg Streptomycin, 25 μg Amphotericin B, Gibco-Invitrogen, Carlsbad, CA). To induce chondrogenesis, hMSCs were cultured as high-density cell pellets formed by spinning down 1.5×10^6 cells in a 50 ml conical tube, and maintained in chondrogenic medium consisting of serum-free DMEM containing insulin–transferrin–selenious acid mix (ITS) (BD Biosciences, Bedford, MA), 100 μg/ml sodium pyruvate, 40 μg/ml L-proline, 50 μg/ml L-ascorbic acid 2-phosphate, in the presence of 100 nM DEX and 10 ng/ml TGF-β3. After 3 days of culture to allow cell aggregation, the pellets were treated with the indicated concentrations of GlcN. Control cultures consisted of cells incubated in chondrogenic medium (with DEX and TGF-β3). In other experiments, cultures were treated at day 11 with 5 ng/ml IL-1β for 24 h, washed and then incubated for an additional 10 days in chondrogenic medium in the presence or absence of GlcN at the indicated concentrations. The cultures were processed for sGAG assays, RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) analysis, as well as histochemical and immunohistochemical analyses after fixation in 4% paraformaldehyde.

ISOLATION AND CULTURE OF CHONDROCYTES

Normal human articular cartilage was purchased from National Disease Research Interchange (NDRI) (Philadelphia, PA) and OA articular cartilage was obtained from patients undergoing knee arthroplasty at George Washington University according to an IRB approved protocol. To isolate normal and OA chondrocytes, cartilage was shaved off the femoral condyle and the tibial plateau, washed three times with PBS and digested with 0.1% Trypsin–ethylene diamine tetraacetic acid (EDTA) in serum-free culture medium for 1 h at 37°C under agitation. After washing in PBS, the cartilage pieces were minced, and then digested with collagenase 2 (Sigma) at 50 μg/ml in serum-free medium overnight at 37°C under agitation. The chondrocyte suspension was filtered through a 40 μm cell strainer, washed with PBS, and plated at 1.5×10^5 cells/cm^2 in DMEM containing 10% FBS and antibiotic–antimycotic solutions. Cells were allowed to adhere, spread and proliferate for 7–10 days. Normal and OA chondrocytes were used for experiments between P1 and P2.

Primary chondrocytes were cultured as either monolayer or high-density pellets and maintained in either control medium (high glucose DMEM with 10% FBS) or in defined medium (high glucose DMEM, ITS, sodium pyruvate, L-proline and L-ascorbic acid 2-phosphate). For pellet cultures, cells were

first allowed to aggregate for 24 h after pelleting, and then subjected to the following treatments: (1) treatment with 5 ng/ml IL-1β for 24 h at either day 1 or day 11; and (2) treatment with GlcN for an additional 10 days (after IL-1β treatment at day 11) or 20 days (after IL-1β treatment on day 1). Monolayer cultures were grown in control medium (high glucose DMEM with 10% FBS) for 24 h, and then treated with GlcN for 3, 7 and 11 days. Harvested cultures were processed for RNA isolation followed by RT-PCR, histochemical and immunohistological analyses, as described above.

RT-PCR ANALYSIS OF GENE EXPRESSION

Total RNA was extracted from monolayer and pellet cultures using the Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was done using 0.5−1 μg RNA and a one step RT-PCR was carried out according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Reverse transcription reactions were performed at 50°C for 30 min and terminated by incubation at 94°C for 2 min. PCR was carried out for 40 cycles of amplification, consisting of 94°C for 30 s, 55−60°C for 30 s and 72°C for 45 s. For real-time RT-PCR analyses, total RNA (1−5 μg) was reverse transcribed using Invitrogen's first strand DNA synthesis kit. Real-time PCR reactions were carried out using 10 ng of cDNA and Syber Green mix (Biorad Laboratories, Hercules, CA). Reactions were carried out for 40 cycles of amplification, consisting of denaturation at 94°C for 30 s, annealing at 57−60°C for 30 s and elongation at 72°C for 30 s. To control for reaction specificity, PCR products were melted for 1 min, re-annealed, and then amplified for 18−20 cycles at Tm + 1°C increment at each cycle. Quantitative analyses were performed using BioRad iCycler software, and standard curves were generated using 10-fold serial dilutions of cDNA with a correlation coefficient of ≥0.9 and a PCR efficiency of ≥80%. Specific primers for aggrecan, collagen II, MMP-13, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were designed based on their corresponding GenBank cDNA sequence. Primer sequences used in semiquantitative and real-time PCR and predicted size of the PCR products are presented in Table I.

HISTOLOGICAL AND IMMUNOHISTOCHEMICAL ANALYSES

Pellet cultures were fixed in 4% paraformaldehyde, dehydrated using a graded series of ethanol washes, and embedded in paraffin. Sections of 8 μm in thickness were stained with alcian blue. For immunohistochemical analyses, sections were pre-digested with hyaluronidase (Sigma) in 10 mM Tris−HCl, pH 7.5, for 30 min at 37°C, then incubated overnight at room temperature with the indicated antibodies in Tris buffered saline (TBS; pH 7.4) containing 0.1% BSA. Specific antibodies to aggrecan and collagen II were obtained from Developmental Studies Hybridoma Bank (Iowa City, IA). Immunostaining was detected by using the chromogenic Streptavidin-Peroxidase Histostain SP Kit for DAB (Zymed Laboratories, San Francisco, CA). Cells were examined using a Leica microscope at the indicated magnification.

sGAG ASSAY

Pellet cultures were digested overnight at 60°C with 300 μg/ml papain (Sigma) in 20 mM sodium phosphate buffer, pH 6.8, containing 5 mM EDTA and 2 mM dithiothreitol (DTT). Cell lysates were cleared by centrifugation and sGAG was determined using the Blyscan sGAG assay kit (Biocolor Ltd, UK) according to the manufacturer's procedure. Briefly, cell lysates were incubated with the dye reagent 1,9-dimethylmethylene blue (DMMB), for 30 min, and unbound dye removed by centrifugation. Bound dye was dissociated from the sGAG−dye complex and quantified spectrophotometrically based on A_{656}. Using chondroitin 4-sulfate as a standard, total sGAG was determined and was expressed as a function of protein content.

WESTERN BLOT ANALYSIS

Harvested cultures were washed with cold PBS, and lysed in a buffer containing 0.1% NP-40, 10 mM Tris−HCl, pH 7.9, 10 mM $MgCl_2$, 15 mM NaCl, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), and protease inhibitor cocktail (Roche, Indianapolis, IN) to isolate cytoplasmic proteins. Cell extracts (25−50 μg protein) were resolved by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred to nitrocellulose. After transfer, the filters were blocked for 1 h with 2% BSA−TBS−0.1% Tween-20, incubated with MMP-13 antibody (R&D systems, Minneapolis, MN) or β-actin antibody (Sigma), followed by 1-h incubation with a horseradish peroxidase-labeled goat

Table I

Primer	Sequence	Annealing (°C)	Product size (bp)
Semiquantitative PCR primers			
Aggrecan (F)	5′-TGA CCA CTT TAC TCT GGG TTT TCG-3′	57	350
Aggrecan (R)	5′-ACA CGA TGC CTT TCA CCA CG-3′		
Col II (F)	5′-CCG CGG TGA GCC ATG ATT CG-3′	57	377 (IIA)
Col II (R)	5′-CAG GCC CAG GAG GTC CTT TGG G-3′		171 (IIB)
MMP-13 (F)	5′-ACT TTA TGC TTC CTG ATG ACG ATG-3′	57	590
MMP-13 (R)	5′-TGC TGT ATT CAA ACT GTA TGG GT-3′		
G3PDH (F)	5′-ACC ACA GTC CAT GCC ATC AC-3′	59	450
G3PDH (R)	5′-TCC ACC ACC CTG TTG CTG TA-3′		
Real-time PCR primers			
Aggrecan (F)	5′-CAC GAT GCC TTT CAC CAC GAC-3′	57	181
Aggrecan (R)	5′-TGC GGG TCA ACA GTG CCT ATC-3′		
Collagen IIB (F)	5′-GGA AAC TTT GCT GCC CAG ATG-3′	59	167
Collagen IIB (R)	5′-TCA CCA GGT TCA CCA GGA TTG C-3′		
MMP-13 (F)	5′-AAC GCC AGA CAA ATG TGA CCC-3′	57	120
MMP-13 (R)	5′-TCC GCA TCA ACC TGC TGA GG 3′		
G3PDH (F)	5′-CAA GGC TGA GAA CGG GAA GC-3′	57	194
G3PDH (R)	5′-AGG GGG CAG AGA TGA TGA CC-3′		

anti-rabbit antibody (diluted 1:20,000) (Amersham Biosciences, Piscataway, NJ). Immunoreactive protein bands were detected using a chemiluminescence detection system (Pierce Biotechnology, Rockford, IL).

Results

LOW DOSES OF GLCN ENHANCE CHONDROGENESIS OF hMSCs

High-density pellet cultures of hMSCs undergoing chondrogenesis were treated with GlcN at various concentrations to test the effect on the expression of markers of chondrogenic phenotype. Chondrogenesis was induced for 21 days by maintaining the cultures in a chondrogenic medium containing 100 nM DEX and 10 ng/ml of TGF-β3 in the absence or presence of 50 μM–20 mM GlcN. In initial experiments, it was found that the addition of GlcN during the early steps of high-density pellet formation disrupted the integrity of the cell pellet, and resulted in lack of chondrogenic marker gene expression (data not shown). Consequently, to assess the effect of GlcN on chondrogenesis, GlcN treatment was started at day 4 after the cell pellet was well formed, and was continued through day 21. To quantify the effect of GlcN on chondrogenesis of hMSCs, we carried out real-time RT-PCR analysis of total RNA prepared from day 21 hMSC pellet cultures maintained in chondrogenic medium containing DEX and TGF-β3, in the presence or absence of 100 μM, 1 mM or 10 mM GlcN. As shown in Fig. 1, expression of the cartilage matrix genes, aggrecan and collagen II (II B RNA isoform) was induced in control cultures maintained in chondrogenic medium, indicating the induction of a chondrogenic phenotype in the hMSC cultures under these experimental conditions. As shown in Fig. 1 (left panel), expression of aggrecan in pellet cultures treated with 1 mM GlcN was enhanced by over 30% compared to control untreated pellets. Similar upregulation of collagen II gene expression was observed in the presence of 1 mM GlcN [Fig. 1(B), right panel]. However, treatment with 10 mM GlcN resulted in the inhibition of both aggrecan and collagen II gene expression relative to untreated control. Physiological plasma GlcN concentrations ranged from 150 μM to 300 μM, in human adults ingesting a typical dose of GlcN supplement of 1500 mg/day (20 mg/kg in a 75 kg subject)[25]. These results showed that GlcN concentrations within the range achieved physiologically with oral dosing of GlcN enhanced cartilage specific cartilage ECM gene expression during chondrogenesis of hMSCs, while higher doses were inhibitory.

We next examined by immunohistochemistry the effect of GlcN treatment on aggrecan and collagen II protein in sections of day 21 hMCS pellet cultures maintained in chondrogenic medium. In control cultures, cartilage phenotype was demonstrated by immunostaining of aggrecan and collagen II (Fig. 2). Treatment of hMSCs undergoing chondrogenesis with 100 μM or 1 mM GlcN did not appear to affect collagen II levels, but substantially enhanced aggrecan protein level as shown by immunostaining (Fig. 2). Biochemical analysis of cultures treated with 100 μM GlcN contained significantly higher levels of sGAG levels as compared to untreated control, while 10 mM GlcN treatment was inhibitory. However, no significant increase in sGAG was observed when cells were treated with 1 mM GlcN (Fig. 3). This discrepancy between the RNA and aggrecan/sGAG levels at 1 mM GlcN could result from the spatial heterogeneity of differentiation under these conditions; i.e., intense aggrecan immunostaining in the periphery of the pellet at a higher level than that of the control, but reduced aggrecan immunostaining in the center of the pellet when compared to the cultures treated with 100 μM GlcN and control (Fig. 2). Taken together, these results showed that GlcN concentrations within the range achieved physiologically with oral dosing of GlcN enhanced production of cartilage specific matrix components during chondrogenesis of hMSCs.

GLCN IS EFFECTIVE IN MAINTAINING CHONDROGENIC PHENOTYPE

We next examined whether the pro-chondrogenic effect of GlcN also applied to primary human chondrocytes. Treatment of chondrocyte pellet cultures with various doses of GlcN showed that doses ranging from 50 μM to 10 mM GlcN enhanced or maintained collagen II and aggrecan gene expression, but doses higher than 10 mM were inhibitory (data not shown). To further characterize the effect of GlcN on the maintenance of chondrocyte differentiation markers, we performed a time course analysis of collagen II and aggrecan gene expression in both monolayer and high-density pellet cultures of chondrocytes maintained in serum-containing medium supplemented with ascorbate and treated with 10 mM GlcN for up to 11 days. RT-PCR analysis of monolayer cultures showed that expression of

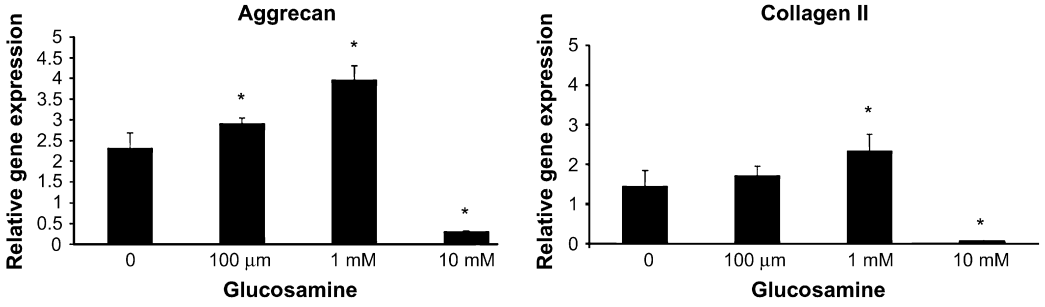

Fig. 1. Effect of GlcN on expression of cartilage matrix gene expression during chondrogenic differentiation of hMSCs. High-density pellet cultures of hMSCs undergoing chondrogenesis in defined medium supplemented with DEX and TGF-β were co-treated with various doses of GlcN from day 11 through day 21 and total RNA was subjected to quantitative RT-PCR analysis of cartilage marker gene expression with G3PDH as an internal control. Levels of specific gene expression (expressed as average copy number relative to that of G3PDH) show that treatment with 100 μM and 1 mM GlcN significantly ($P \leq 0.001$) enhanced both aggrecan (left) and collagen II (right) gene expression, while treatment with 10 mM GlcN was inhibitory ($P \leq 0.05$) for both genes in hMSCs undergoing chondrogenesis. The data represent the mean ± SD (*$P \leq 0.05$) as determined by ANOVA, of three independent experiments performed in triplicate.

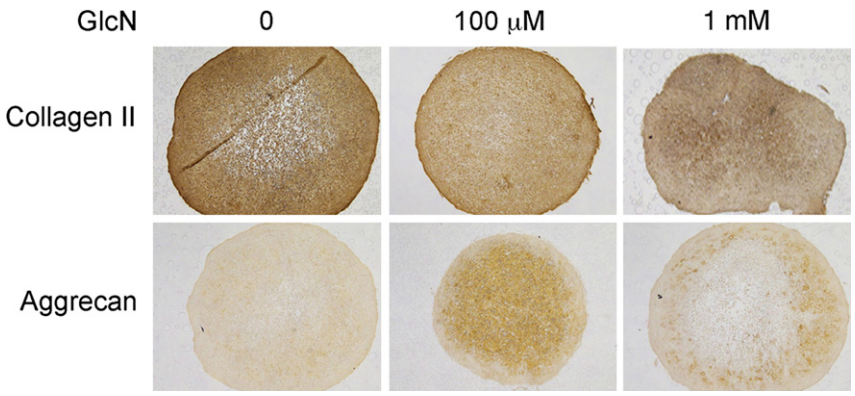

Fig. 2. Immunohistochemical analysis of GlcN regulation of cartilage marker gene expression in pellet cultures of hMSCs. Day 21 cultures grown in the presence of DEX and TGF-β and treated in the presence of the indicated amount of GlcN were fixed and sections were immunostained for aggrecan and collagen II. While GlcN treatment did not appear to affect collagen II staining, aggrecan levels were increased by 100 μM GlcN and to a lower extent by 1 mM GlcN treatment.

both aggrecan and collagen II (IIB mRNA isoform) was downregulated in a time-dependent manner. In comparison, under high-density pellet cultures conditions, untreated primary chondrocytes maintained expression of aggrecan and collagen IIB up to day 7 and expressed higher levels of collagen IIA mRNA isoform [Fig. 4(A)]. After day 7, aggrecan and collagen IIA levels decreased while collagen IIB remained unchanged. Interestingly, GlcN treatment partially reversed the decrease in aggrecan in both monolayer and pellet cultures and further maintained the levels of collagen IIA mRNA in pellet cultures [Fig. 4(A)].

Immunohistochemistry of collagen II showed low levels of staining in monolayer cultures and, in pellet cultures, increased levels of collagen II were detected in sections of GlcN-treated cultures, when compared to untreated cultures. In addition, alcian blue staining of sulfated proteoglycans was increased in monolayer cultures treated with GlcN when compared to untreated controls, and was maintained at equivalent levels in pellet cultures grown in the presence or absence of GlcN [Fig. 4(B)]. Thus, GlcN treatment maintained cartilage ECM marker gene expression and the levels of matrix components in chondrocyte cultures grown in the presence of serum *in vitro*.

GLCN BLOCKS IL-1β-INDUCED INHIBITION OF MATRIX GENE EXPRESSION IN OA CHONDROCYTES

To assess the chondroprotective activity of GlcN, high-density pellet cultures of human OA chondrocytes were grown in defined medium containing ITS, treated with 5 ng/ml IL-1β for 24 h at day 1, then exposed to varying doses of GlcN for additional 10 days, and cartilage marker gene expression analyzed by quantitative RT-PCR. As shown in Fig. 5(A), treatment with IL-1β inhibited both aggrecan and collagen II (IIB) basal gene expression. Expression of aggrecan was significantly enhanced by 100 μM and 1 mM GlcN treatment post-IL-1β exposure. In contrast, GlcN treatment alone at 100 μM enhanced collagen II (IIB) levels but provided little protection for the effect post-IL-1β treatment [Fig. 5(A)]. However, treatment with 10 mM GlcN, either with or without IL-1β, resulted in a significant inhibition of aggrecan and collagen II gene expression relative to untreated control [Fig. 5(A)]. Taken together, this data showed that treatment of chondrocyte pellet cultures with 100 μM and 1 mM GlcN, post-IL-1β exposure, maintains collagen II and stimulates aggrecan and gene expression.

Biochemical analysis showed increased levels of sGAG in cultures treated with 100 μM GlcN as compared to untreated control, while 10 mM GlcN treatment was inhibitory. No significant change in sGAG was seen when cultures were treated with 1 mM GlcN [Fig. 5(B)]. Aggrecan immunohistochemistry and alcian blue staining of sections of OA chondrocyte pellet cultures showed that treatment with 100 μM and 1 mM GlcN enhanced aggrecan and sulfated proteoglycans levels, while 10 mM GlcN treatment was inhibitory [Fig. 5(C)]. Taken together, these results suggest that exposure of OA chondrocytes to GlcN concentrations within the range achieved physiologically with oral dosing of GlcN, enhanced aggrecan gene expression and overcame the effect of prior treatment with IL-1β, while high doses of GlcN were deleterious to the chondrocyte phenotype.

GLCN SUPPRESSES IL-1β-INDUCED MMP-13 EXPRESSION IN BOTH OA CHONDROCYTE AND hMSCs PELLET CULTURES

We next tested the effect of GlcN on IL-1β-mediated expression of downstream effectors of cartilage degradation,

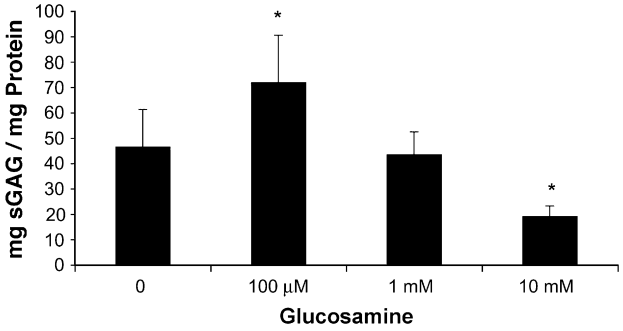

Fig. 3. Effect of GlcN treatment on sGAG content in hMSCs undergoing chondrogenesis. Day 21 pellet cultures grown in the presence of DEX and TGF-β and treated with the indicated amount of GlcN were digested with papain overnight, and sGAG content was determined spectrophotometrically in cleared total cell lysates using a Blyscan assay; with chondroitin 4-sulfate as a standard, and was normalized to total protein content. Results show that 100 μM GlcN significantly ($P \leq 0.001$) enhanced sGAG levels, while treatment with 10 mM GlcN was inhibitory ($P \leq 0.05$) to the accumulation of sGAG in hMSCs undergoing chondrogenesis. The data represent the mean ± SD ($^*P \leq 0.05$), as determined by ANOVA, of two independent experiments performed in triplicate.

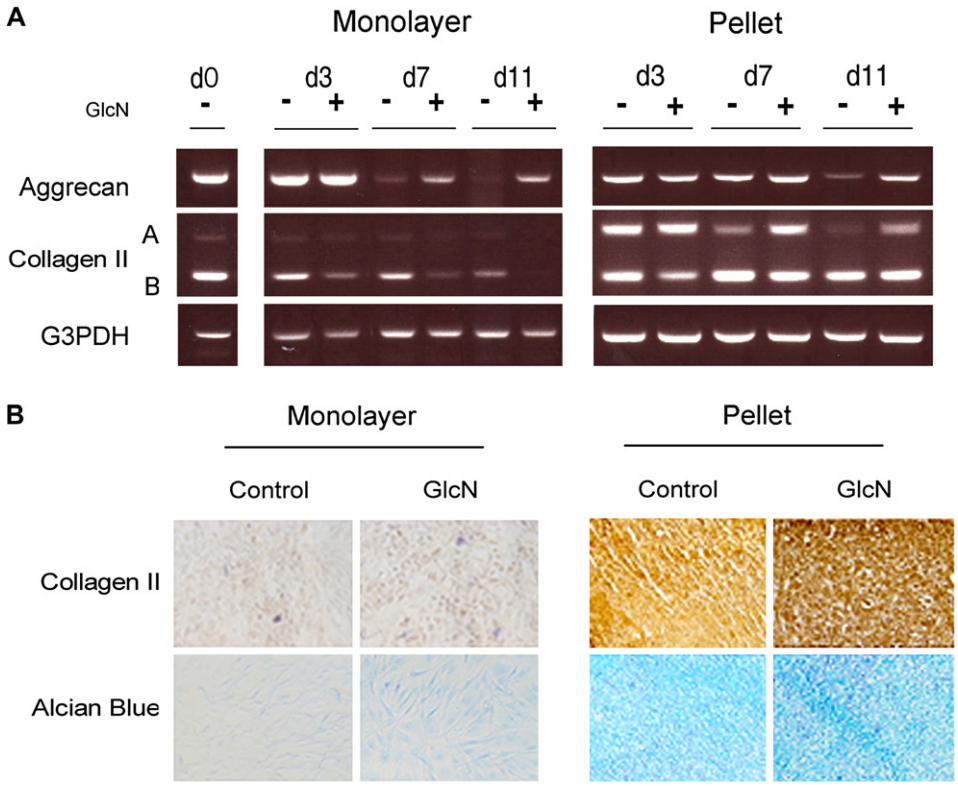

Fig. 4. Effect of GlcN on cartilage matrix gene expression in human chondrocytes grown in the presence of serum. Human chondrocytes were grown for 3–11 days as a monolayer or pellet culture in serum-containing medium supplemented with ascorbate and treated in the presence or absence of 10 mM GlcN. (A) RT-PCR. Results show that GlcN blocks the time-dependent downregulation of aggrecan in both monolayer and pellet cultures and enhanced collagen II expression in pellet cultures only. (B) Immunohistochemistry and alcian blue staining. Chondrocyte monolayer culture (day 7) and sections of pellet culture (day 11) were immunostained for collagen II or directly stained with alcian blue. Results show that GlcN enhanced alcian blue staining in monolayer culture and increased collagen II levels in pellet culture.

such as MMP-13, in pellet cultures of chondrocytes and hMSCs. Normal and OA chondrocytes were grown in defined medium, treated with or without 5 ng/ml IL-1β for 24 h on day 1, and then exposed to GlcN for one additional day or 10 days. hMSCs undergoing chondrogenesis were treated on day 11 with IL-1β for 24 h, and the cultures maintained for additional 10 days, up to day 21, in the presence or absence of varying doses of GlcN. RT-PCR analysis showed that both OA chondrocytes and MSCs expressed MMP-13, particularly the former, and that treatment with IL-1β enhanced their basal levels of MMP-13. On the other hand, IL-1β treatment induced a strong expression of MMP-13 in normal chondrocytes, which originally did not express any MMP-13 [Fig. 6(A)]. Following 10 mM GlcN treatment, this upregulation of MMP-13 expression was completely blocked in normal chondrocytes and partially reversed in OA chondrocytes and hMSCs [Fig. 6(A)]. In hMSCs, quantitative RT-PCR analysis showed that following IL-1β exposure, treatment with 100 μM GlcN inhibited MMP-13 expression by two-fold, which was however upregulated by a 10 mM dose of GlcN. In contrast, in OA chondrocytes, GlcN treatment inhibited MMP-13 expression in a dose-dependent manner [Fig. 6(B)]. Western blot analysis of proteins extracted from OA chondrocytes treated with IL-1β showed that subsequent GlcN treatment reduced latent and active MMP-13 protein levels in a dose-dependent manner [Fig. 6(C)]. Taken together, these results suggest that GlcN blocks the effects of pro-inflammatory mediators on both chondrocytes and hMSCs undergoing chondrogenesis, by reducing the expression of matrix degrading enzymes, such as MMP-13, thereby preventing cartilage matrix damage.

Discussion

While GlcN salts have been considered a new class of drugs with putative chondroprotective activity, their mechanism of action is still not well understood and their benefit is still controversial, due in part to the small sample size and short-term follow-ups of clinical studies[11–13,21,26]. In this study, we have analyzed the *in vitro* effects of GlcN on mesenchymal chondrogenesis and on the action of the pro-inflammatory cytokine, IL-1β on chondrocytes and a multipotential hMSC cell line undergoing chondrogenesis. We have selected to use D (+) GlcN-HCl because it is the major circulating GlcN form *in vivo*. The sulfated form of GlcN found in dietary supplements is modified to GlcN-HCl upon ingestion. Our studies showed that low doses (100 μM, 1 mM) of GlcN enhanced differentiation of a multipotential hMSC cell line undergoing chondrogenesis, by upregulating expression of cartilage matrix markers. These cells were established from trabecular bone and express the cell surface antigens Stro-1, CD 73 and CD105[27,28]. These cells give rise to a homogenous population of stable proliferating progenitor cells which display an undifferentiated phenotype demonstrated by the absence of expression of collagen II or aggrecan, although some collagen I RNA can be detected[22–24,29]. To validate the observed effects on hMSCs in

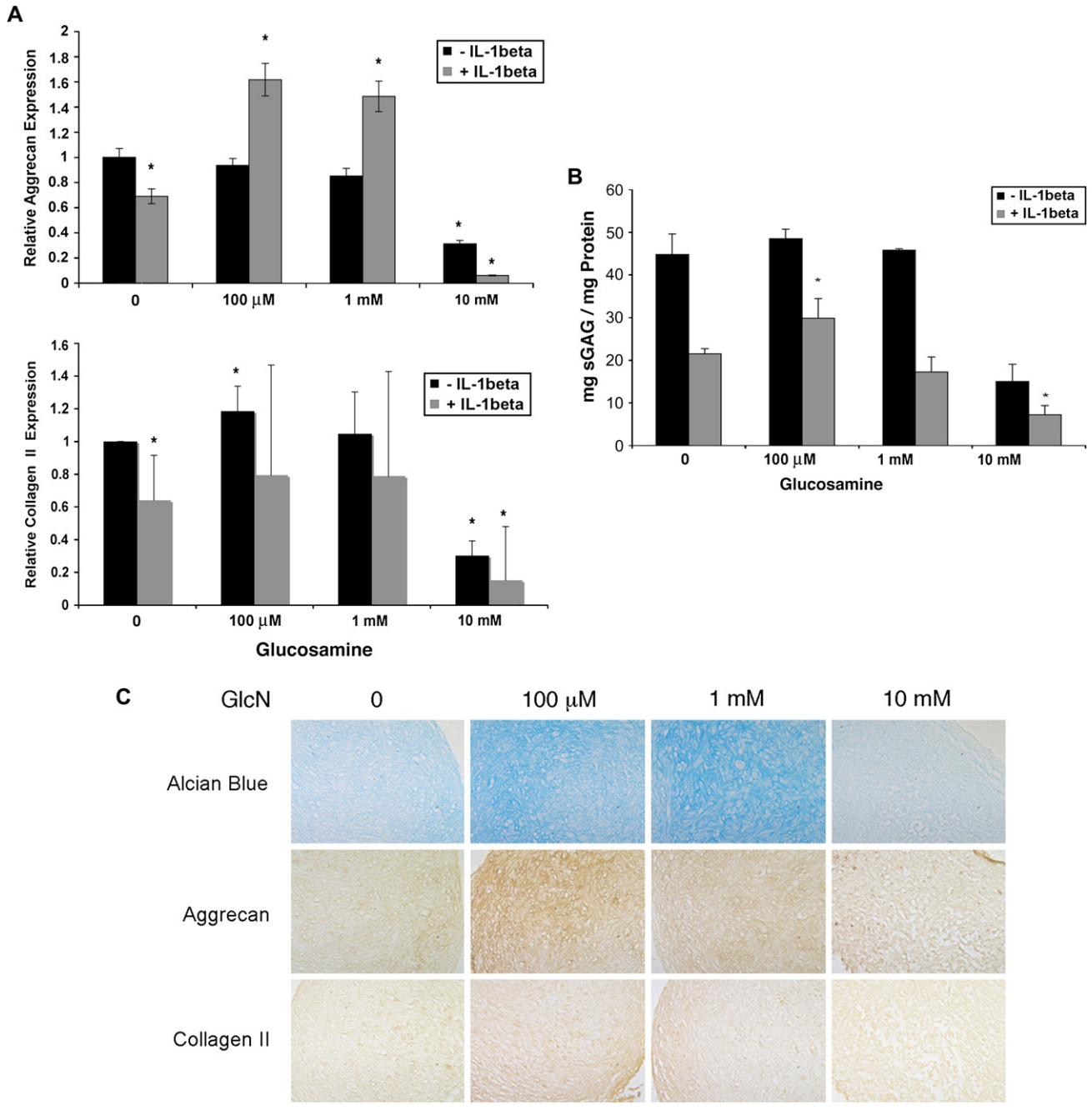

Fig. 5. Effect of GlcN on cartilage matrix gene expression in human chondrocytes challenged with IL-1β. Human OA chondrocytes were grown as pellet cultures in defined medium, treated with IL-1β for 24 h, then washed and treated in the presence or absence of the indicated amounts of GlcN for additional 10 days. (A) Quantitative RT-PCR. Results of specific gene expression (expressed as average copy number relative to that of G3PDH) show that in the absence of GlcN treatment, IL-1β inhibits both aggrecan (top) and collagen II (bottom) gene expression. Treatment with 100 μM and 1 mM GlcN significantly ($P \leq 0.001$) enhanced aggrecan gene expression and maintained collagen II expression post-IL-1β treatment, while treatment with 10 GlcN was inhibitory ($P \leq 0.05$). The data represent the mean ± SD, of five independent experiments performed in triplicate. (B) Day 11 pellet cultures grown as in (A) were digested with papain overnight and sGAG content was determined. Results show that post-IL-1β treatment, 100 μM GlcN significantly ($P \leq 0.001$) enhanced sGAG content while treatment with 10 mM GlcN suppressed ($P \leq 0.05$) sGAG accumulation. The data represent the mean ± SD (*$P \leq 0.05$), as determined by ANOVA, of two independent experiments performed in triplicate. (C) Immunohistochemistry. Pellet cultures of OA chondrocytes grown as described in (A) were fixed and sections were immunostained for aggrecan and collagen II or directly stained with alcian blue for sulfated proteoglycans. Collagen II and aggrecan protein levels and alcian blue staining of sulfated proteoglycans were increased by 100 μM and 1 mM GlcN, and were unaffected or inhibited by 10 mM GlcN, respectively.

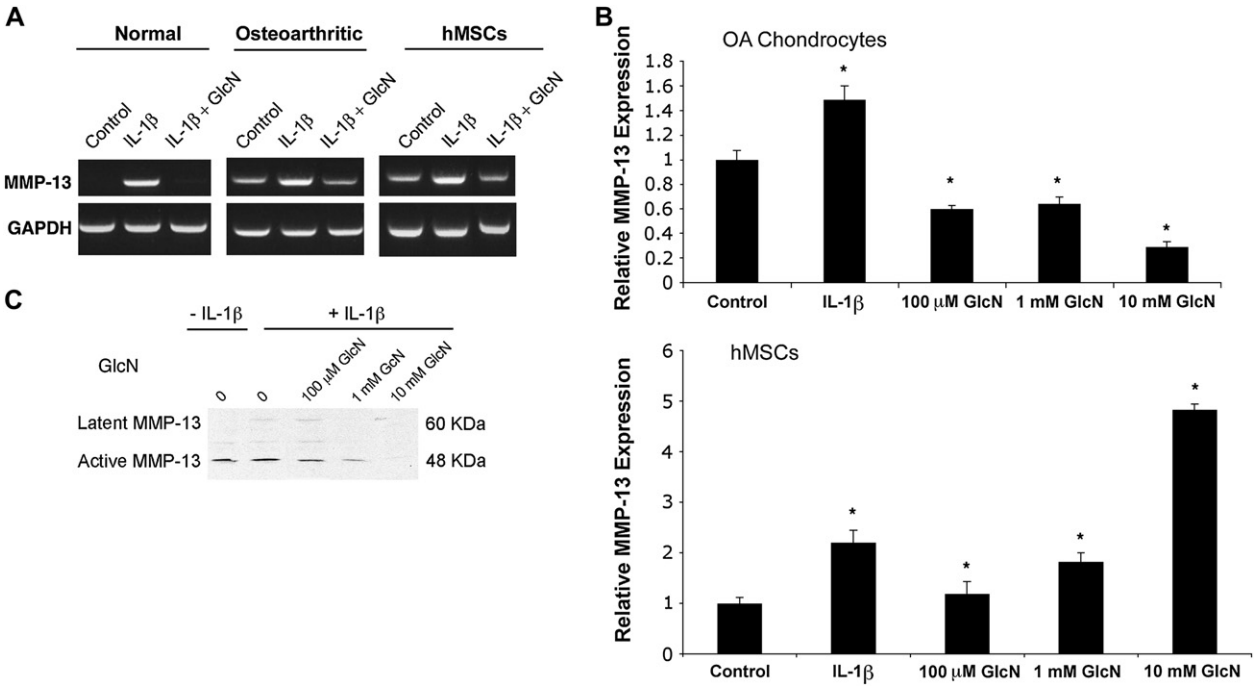

Fig. 6. GlcN suppression of IL-1β-induced MMP-13 expression in chondrocytes and hMSCs. (A) RT-PCR. Normal and OA human chondrocytes were cultured in high-density pellet cultures in ITS medium, and hMSCs were induced to undergo chondrogenesis as described above, and treated with 5 ng/ml IL-1β for 24 h at day 1 or day 11, respectively, followed by treatment for an additional 10 days in the presence or absence of 10 mM GlcN. The data show that GlcN inhibited IL-1β-induced MMP-13 expression in both OA and normal chondrocytes as well as in hMSCs undergoing chondrogenesis. (B). Quantitative RT-PCR. Results from OA chondrocytes show that treatment with GlcN at all concentrations inhibited MMP-13 gene expression, while in hMSCs, suppression was seen at 100 μM GlcN, with increase seen at 10 mM GlcN. The data represent the mean ± SD ($^*P \leq 0.05$) of five independent experiments each performed in triplicate. (C) Western blot. Cell extracts (50 μg protein) prepared from OA chondrocytes grown and treated as in (B) were analyzed by immunoblotting for MMP-13. Results show that GlcN treatment downregulated basal active MMP-13 levels and inhibited IL-1β-induced MMP-13 expression. A β-actin blotting was used as a control for equal protein loading (not shown).

general, we have also examined the effect of GlcN on primary human bone marrow stromal cells. Based on quantitative RT-PCR and sGAG analyses, we found that both 100 μM and 1 mM GlcN treatment enhanced the levels of aggrecan mRNA and increased sGAG content (unpublished observation). To our knowledge, this is the first study to examine the effects of GlcN on hMSCs; this chondro-enhancing effect could contribute toward improving cartilage repair and homeostasis mediated by adult stem cells.

Physiological plasma GlcN concentrations in human adults ingesting a typical dose of GlcN supplement of 1500 mg/day (20 mg/kg in a 75 kg subject), ranged from 150 μM to 300 μM[25] and reached the 10 mM range after repeated doses[30]. Our results showed that maximal enhancement of the chondrogenic phenotype by GlcN treatment was achieved at 100 μM, while higher doses were in fact inhibitory to both MSCs and chondrocytes. Others have also reported similar protective effects at physiological doses of GlcN, and chondrocyte toxicity has been associated with GlcN concentrations exceeding 10 mM *in vitro*[18,25,31].

The pro-inflammatory cytokine, IL-1β, induces a large cascade of events that leads to cartilage damage. In preliminary studies, we have examined the effect of GlcN treatment prior to IL-1β exposure in the presence or absence of GlcN. We observed that GlcN pre-treatment did not block the transient inhibitory effect of the 24-h IL-1β treatment, on markers of chondrogenic phenotype. However, GlcN pre-treatment is protective if the cells were allowed to recover for 9 days in the presence or absence of GlcN (unpublished data). Our studies showed that treatment of chondrocyte pellet cultures with GlcN at physiological doses of 100 μM, post-IL-1β exposure, inhibits expression of MMP-13. GlcN treatment was also able to reduce gene expression levels of the aggrecanases MMP-3 and A disintegrin and metalloproteinase with thrombospondin motif-5 (ADAMTS-5), in both chondrocytes and MSCs (unpublished data) suggesting that the protective effects of GlcN may be mediated in part through downregulation of degrading enzymes that target both collagen II and aggrecan, two major components of the cartilage matrix. Other studies have shown that GlcN blocks other catabolic events such as IL-1β-induced nuclear factor κ beta (NFκB) activation, NO production, and cyclooxygenase 2 (Cox-2) expression[18,32]. These effects may account for our observation of the GlcN effect on the maintenance of the differentiated phenotype of chondrocytes, grown *in vitro* in the presence of serum, which normally would gradually lose cartilage matrix gene expression as a function of time.

Although low doses of GlcN lack toxicity and inhibition of MMP-13 expression and cartilage degradation showed dose dependence, higher doses of GlcN, e.g., 10 mM, are inhibitory to hMSC chondrogenesis and phenotype maintenance of chondrocytes grown in defined medium conditions. High doses of GlcN have been shown to impair metabolic activity leading to cell death in bovine chondrocytes[31]. In contrast, high doses of *N*-acetyl GlcN did not induce chondrocyte cell death *in vitro*, and intra-articular injection of *N*-acetyl GlcN in rabbit with experimental OA was

shown to have chondroprotective and anti-inflammatory activity[17,33]. Similar protection against pain was reported in a recent trial of human OA, with a GlcN/chondroitin combination[16].

In addition to its chondroprotective effect, our studies showed that treatment of chondrocyte pellet cultures with GlcN at physiological doses of 100 μM, post-IL-1β exposure, stimulates aggrecan expression and maintains collagen II expression. Others have also shown GlcN to induce proteoglycan synthesis and enhance sulfate incorporation. In an animal study, GlcN treatment was shown to result in a significant increase in sGAG content, without change in collagen levels, in damaged knee joints of rabbits injected with chymopapain, whereas no effect of GlcN on sGAG content in normal cartilage was found[34]. *In vitro*, GlcN treatment of human OA chondrocytes treated with retinoic acid, IL-1β or grown in suspension under constant agitation, enhanced aggrecan protein levels without any effect on collagens[18,35–37]. Taken together, these results suggest that GlcN may act by controlling inflammatory mediators and stimulating proteoglycan synthesis in response to cartilage damage. Our findings are thus, in general agreement with these published observations.

In conclusion, our data suggest that GlcN enhances the chondrogenic phenotype of hMSCs by upregulating expression of aggrecan and collagen II. In addition, GlcN promotes the maintenance of the chondrocyte phenotype by enhancing sulfated proteoglycan accumulation, and reduces cartilage matrix degradation by inhibiting IL-β-induced MMP-13 expression. Enhancing hMSC chondrogenesis in combination with blocking cartilage degradation may contribute toward the chondroprotective properties of GlcN observed in many studies. Understanding the mechanism of action of GlcN on the differentiation of chondroprogenitor cells and the activities of chondrocytes should provide a rational basis for the development of chondroprotective therapies for degenerative joint diseases.

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